RNA-Affinity Chromatography

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[Abstract] RNA-affinity chromatography assays are used to identify proteins binding specific RNA sequences. These proteins represent potential factors contributing to the function of RNA molecules. In our lab, we have used this protocol to identify proteins binding sequence motifs involved in replication and transcription of positive strand RNA viruses. The assay described in this protocol consists on the immobilization of 5'-biotinylated RNA oligonucleotides (30-40 nt) on a streptavidin-conjugated, paramagnetic solid matrix. Then, cytoplasmic protein extracts pre-cleared on the solid matrix to decrease nonspecific binding, were incubated with the immobilized RNA molecules in the presence of a nonspecific competitor. RNA-protein complexes immobilized on the paramagnetic solid matrix were isolated using a magnet and the bound proteins were separated by polyacrylamide gel electrophoresis for proteomic analysis.

Materials and Reagents

1. 5'-biotinylated RNAs (Sigma-Aldrich)
2. Streptavidin conjugated solid matrix (Dynabeads M-280 Streptavidin) (Life Technologies, Invitrogen™, catalog number: 11205D)
3. Protease inhibitor (Complete Protease Inhibitor Cocktail Tablets) (Roche, catalog number: 1697498)
4. 10% glycerol
5. Non-specific competitor tRNA (Baker yeast) (Sigma-Aldrich, catalog number: R8759)
6. NuPAGE LDS sample buffer (Life Technologies, Invitrogen™)
7. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D9779)
8. Bis-Tris-Gel (Life Technologies, Invitrogen™)
9. NuPAGE MOPS SDS running buffer (Life Technologies, Invitrogen™)
10. Coomassie Simply Blue Safe Stain (Life Technologies, Invitrogen™, catalog number: LC6060)
11. Diethylpyrocarbonate (DEPC) treated water
12. RNase inhibitor (Rnasin) (Promega Corporation, catalog number: N2611)
13. IGEPAL CA-630 (NP-40 substitute) (Sigma-Aldrich, catalog number: I3021)
14. KCl

15. Glycerol
16. NP-40
17. EDTA
18. Extraction buffer (see Recipes)
19. H-BW solution (see Recipes)
20. BW solution (see Recipes)

Equipment

1. 150 mm plates
2. Protein gel cassettes
3. Orbital Shaker (J.P. Selecta, catalog number: Orbit 3000445)
4. Magnetic particle concentrator for microcentrifuge tubes (Dynal Biotech, Dynal MPC-S 120.20)

Software

1. Image Lab V3.0 (Bio-Rad Laboratories)

Procedure

1. The capture of proteins binding specific RNA was performed using 5'-biotinylated RNAs linked to a streptavidin conjugated solid matrix.
2. Proteins were extracted from transmissible gastroenteritis virus (TGEV) infected human Huh7 cells using an extraction buffer containing 10% Igepal (NP-40) detergent. The cell lysate was then centrifuged to pellet the nuclei and save the cytoplamic extract. Briefly, Huh7 cells grown on 150 mm plates were infected with TGEV virus. At 48 h post infection, cells from two plates were harvested and resuspended with 500 µl extraction buffer without Igepal (NP-40) on ice. Cellular extracts were incubated for 15 min on ice and then, Igepal (NP-40) detergent was added to the cell suspension to a final concentration of 10%. Extracts were mixed by vortexing, incubated for 10 additional minutes on ice and centrifuged for 2 min at maximum speed to recover the supernatant. Protein extracts may be storaged at -80 °C with 10% glycerol.
3. 60 µl of streptavidin-conjugated solid matrix (10 µg/µl) were used per RNA binding assay. Before binding to RNA, the solid matrix was washed twice with 360 µl of solution H-BW. All washes were performed by inverting the tube. No incubation time was required. After

washing, the solid matrix was separated from the supernatant using a magnet. Leave the tubes in the magnet for 1-2 min.

4. Pre-clearing protein extracts on solid matrix not bound to RNA. Protein extracts diluted in H-BW solution (500 µg of total protein per RNA binding assay), were precleared three times by incubating with 60 µl of solid matrix in an orbital shaker at 12 rpm, for at least 5 h each time at 4 °C. Separate the solid matrix using a magnet. Leave the tubes in the magnet for 1-2 min. The solid matrix was discarded after each pre-clearing incubation and the supernantant was transferred to a new tube containing 60 µl of new solid matrix. After the third preclearing, the supernatant was preserved for RNA-binding (step 7).
5. RNA-immobilization on the streptavidin solid matrix. For each RNA binding assay, 60 µl of solid matrix were used. Previously, the solid matrix was washed twice with 60 µl of BW solution as a minimum volume. Then, the streptavidin matrix was incubated with the biotinylated RNA (8 µg) in 60 µl of BW solution for 30 min at RT.
6. Immobilized RNAs on the solid matrix were washed twice with 60 µl of H-BW solution as a minimum volume. Remove the wash solution after placing the tubes in a magnet.
7. RNA-protein binding. Add to the immobilized RNAs, 500 µg of precleared protein extract (from step 4) resuspended in H-BW solution and different amounts of non-specific competitor tRNA (0.5 or 1.25 µg tRNAs /µg protein). Incubate the mixture overnight in an orbital shaker at 4 °C and a speed of 12 rpm. The total volume of the sample was 180 µl (three times the solid matrix volume) consisting of 120 µl of precleared protein extract, 25 µl of tRNAs and 35 µl of H-BW. All the solutions were prepared in DEPC-water in the presence of 0.4 U/µl of RNAse inhibitor to minimize RNA degradation.
8. Place the tubes in a magnet and remove the supernatant containing non-bound proteins. Wash three times with 120 µl H-BW solution.
9. Elute the proteins bound to immobilized RNAs with NuPAGE LDS sample buffer supplemented with DTT 100 mM for 10 min at RT.
10. Proteins bound to RNAs were resolved in NuPAGE 4-12% Bis-Tris-Gel by electrophoresis with NuPAGE MOPS SDS running buffer. Finally gels were stained with Coomassie Simply Blue Safe Stain. Images were taken with Image Lab V3.0.

Recipes

1. Extraction buffer
   2.5 mM HEPES (pH 7.9)
   2.5 mM KCl
   25 µM EDTA
   0.25 mM DTT

Protease inhibitor: One tablet diluted in 25 ml extraction buffer.
Igepal is added (if needed) to a final concentration of 10%.

2. H-BW solution
   50 mM HEPES (pH 7.9)
   150 mM KCl
   5% glycerol
   0.01% NP-40
3. BW solution
   5 mM Tris HCl (pH 7.5)
   1 mM EDTA
   1 M NaCl

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References

1. Mateos-Gomez, P. A., Morales, L., Zuniga, S., Enjuanes, L. and Sola, I. (2013). Long-distance RNA-RNA interactions in the coronavirus genome form high-order structures promoting discontinuous RNA synthesis during transcription. *J Virol* 87(1): 177-186.

